2. In the presence of azide the final rate of oxygen uptake was somewhat lower than in its absence, although the length of the induction period was not greatly affected. These cells were not nitrogen-starved.

3. The respiration of suspensions starved of both carbohydrate and nitrogen was not inhibited by azide, but was greatly stimulated by small quantities of ammonium chloride. This stimulation was abolished by suitable concentrations of azide and cyanide.

4. Neither the endogenous respiration nor the fermentation of glucose under anaerobic conditions was affected by added ammonium chloride.

5. L-Glutamic acid, L-aspartic acid, L-asparagine, and urea stimulated glucose oxidation, though none so effectively as ammonia; nitrate, nitrite, and hydroxylamine were inactive.

6. The oxidation rates of sucrose, ethanol, acetate and succinate were also stimulated to varying degrees by ammonia, but the stimulation was much weaker in most cases than with glucose; only with sucrose did the stimulation approach that with glucose.

7. In the presence of glucose the ammonia was bound, and after 3 hr. appeared mainly in the insoluble nitrogen fraction; azide inhibited this binding of ammonia.

8. These results are discussed and it is suggested that enzyme adaptation is a possible interpretation of these phenomena consistent with the observed facts.

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# Studies on Yeast Metabolism

## 3. THE INTRACELLULAR LEVEL OF PYRUVATE DURING YEAST FERMENTATION

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The rate of carbon dioxide production during alcoholic fermentation is a direct measure of the velocity of the yeast carboxylase system, i.e. of the reaction:

$$\text{pyruvate} + H^+ \rightarrow \text{acetaldehyde} + CO_2.$$

This is irreversible (Vennesland, 1951); thus, in the steady state during fermentation, the rate of pyruvate decarboxylation is likely to be determined by the substrate concentration at the enzyme surface. Unfortunately, this quantity cannot be directly determined. The amount of pyruvate in unit weight of yeast at any stage during fermentation is, in principle, determinable. This intracellular level of pyruvate may or may not be simply related to the kinetically important concentration at the site of carboxylase action. If it is, then its measurement at different fermentation rates should give information about the kinetics of yeast carboxylase *in vivo*.

Extracellular pyruvate, appearing in the medium during fermentation, has been frequently studied; recently, for example, by Wang, Labbe, Christensen & Cheldelin (1952). A short report on the intracellular level has appeared (Brady, 1950).

## METHODS

Fermentation was carried out at 30°, using 5 g. (wet wt.) of D.C.L. baker's yeast/100 ml.; the suspension was stirred by a stream of gas, usually $N_2$. Yeast was taken as required from a 500 g. block, wrapped in a plastic bag, and stored in the refrigerator. Samples taken daily over 3 weeks from yeast stored in this way varied little in performance, as shown by fermentation rate or levels of pyruvate and inorganic phosphate. In consequence, such determinations are strictly comparable although fermentation rate and pyruvate levels were not measured simultaneously, but in separate experiments.

### *Determination of fermentation rate*

The average fermentation rate over an interval of (usually) 15 min. was determined after absorption of $CO_2$ in 50 ml. approximately N-NaOH, by titration of precipitated $BaCO_3$. Absorption towers were connected to the vacuum supply in such a way that connexion or disconnexion did not affect the gas pressure in the fermentation vessel. The method proved sensitive and reproducible: duplicate determinations of the rate at a definite time after initiation of fermentation differed by about 2%.

Maximum fermentation rate was not attained until 35 min. or so after addition of glucose: thereafter, the rate gradually declined. Of the constituents added to the medium, most had only a minor effect. Phosphate caused a more rapid decline in later stages of glucose fermentation, which could be partially counteracted by the addition of magnesium and ammonium salts. In a medium consisting of 0·2M glucose, succinate buffer pH 4·1 (0·05M succinic acid + 0·025N-NaOH), 0·01M-$KH_2PO_4$, 0·01M-$(NH_4)_2SO_4$, 0·0025M-$MgSO_4$, 0·001M-$CaCl_2$ and 0·001M-$ZnSO_4$, the fermentation rate was almost constant after 30 min., and this medium was consequently particularly suitable for kinetic studies.

### *Determination of extracellular pyruvate*

A sample (about 3 ml.) of yeast suspension was rapidly transferred to a centrifuge tube and centrifuged 3 min. at 3000 rev. min.$^{-1}$ About 30 sec. elapsed between the time of pipetting the sample, which was recorded, and reaching full speed in the centrifuge. A suitable portion of supernatant was delivered into trichloroacetic acid (TCA), the final solution being 4 ml., 0·5M in TCA. This was kept in an ice-bath for analysis of pyruvate by the method of Friedemann & Haugen (1943).

### *Determination of total pyruvate*

(1) TCA extraction: 2 ml. yeast suspension were pipetted into an equal volume of chilled M-TCA. After 10–15 min. in an ice-bath the yeast was centrifuged down and a portion of the supernatant made up to 4 ml. (0·5M in TCA) for analysis. (2) Perchloric acid extraction was similarly performed. (3) Hot-water extraction: the yeast suspension was delivered into 2·5 times its volume of water at 100° (boiling water bath), and after 5 min. cooled, centrifuged and treated as (1).

As Table 1 shows, methods (1) and (3) gave concordant results, whereas method (2) gave values for total pyruvate similar to the extracellular values. (In a fermentation using phosphate buffer as medium, perchloric acid extracts contained pyruvate in amount intermediate between the extracellular value and that in TCA extracts.) Perchloric acid, which is highly dissociated, probably does not enter the cell and destroy enzyme action sufficiently rapidly. Unless otherwise stated, 'total pyruvate' means the value obtained by TCA extraction.

### *Calculation of intracellular pyruvate*

All pyruvate values are expressed per g. fresh weight of yeast; in the case of extracellular pyruvate this means the amount in that volume of medium which contained 1 g. yeast. The volume occupied by the yeast cells in a suspension was taken to be three-quarters of the centrifuged cell volume, as shown by Conway & Downey (1950), whose haematocrit technique was used. Little variation in the yeast cell volume was found in the different fermentation media employed, and it was considered sufficiently accurate to take the volume of extracellular medium in 100 ml. suspension containing 5 g. yeast as 96·0 ml. No correction was applied to the total pyruvate for the volume of the yeast solids, as such correction was within the analytical error. The difference between the total pyruvate and the extracellular pyruvate, as μmoles/g. yeast, at a given time, is taken as the intracellular pyruvate.

Table 1. *Pyruvate extracted from a fermenting yeast suspension by different methods*

Suspension: 5 g. baker's yeast + 10 m-moles glucose per 100 ml. (1), (2), (3) refer to experiments made at 3-day intervals.

| | Total pyruvate (μmoles/g.) | | | | | |
|---|---|---|---|---|---|---|
| | | Trichloroacetic acid extract | | Perchloric acid extract | | |
| Time (min.) | Hot-water extract | | | | | Extracellular pyruvate (μmoles/g.) |
| 1 | 1·0 (2) | 0·7 (1) | — | 0·1 (1) | 0·1 (2) | — |
| 5 | 3·4 | 3·0 | — | 0·8 | 0·9 | — |
| 10 | 5·8 | 5·2 | 5·8 (3) | 1·9 | 2·1 | 2·0 (3) |
| 20 | 8·4 | 8·6 | 8·9 | 3·7 | 4·1 | 4·1 |
| 30 | 11·3 | 11·4 | 12·2 | 7·0 | 7·4 | 5·4 |
| 40 | — | — | 13·7 | — | — | 6·7 |

*Ion-exchange chromatography*

The nature of the material determined in the colorimetric analysis of pyruvate was examined by anion-exchange chromatography on a 2·5 cm. × 1 cm.² Dowex-1 column (acetate form), using 0·1 N acetic acid + 0·05 M sodium acetate as the eluting solvent. From a yeast suspension which had fermented 0·1 M glucose for 30 min. were prepared: (i) a centrifugate, containing the extracellular pyruvate (6·7 μmoles/g.), (ii) a hot-water extract (total pyruvate, 12·1 μmoles/g.), whilst in a recovery experiment (iii) a known amount of pyruvic acid was added to a centrifugate. Material run on to the Dowex column corresponded to 1·25 g. yeast; the eluate was collected in 25 ml. fractions, which were analysed for pyruvate. In each case, the elution curve suggested the presence of only a single compound. 84–100% of the material added was recovered. It is therefore probable that the bulk of the material reacting in the colorimetric method was, in fact, pyruvate. Ion exchange was also employed to obtain, from sodium pyruvate prepared according to Robertson (1942), a solution of pyruvic acid for use as a standard in the colorimetric analysis. Standardization of the solution was by an iodometric method (Wendel, 1931), checked by a gravimetric method using the 2:4-dinitrophenylhydrazone.

## RESULTS

*Intracellular pyruvate and fermentation rate*

Buffers, when used, contained 5 m-moles succinic acid/100 ml. suspension, and either 2·5 m-moles NaOH (pH 4·1) or 7·5 m-moles NaOH (pH 5·5). All constituents except sugar were brought to the temperature of the thermostatic bath, the suspension being stirred by a stream of nitrogen. Fermentation was initiated by adding the required amount of sugar, as a M solution of glucose.

With no sugar present, the intracellular pyruvate (succinate buffer, pH 5·5) was 0·3 μmole/g., rising only to 0·4 after 30 min. incubation. The interpretation of such small figures as pyruvate is doubtful (Brady, 1950). The close relation between intracellular pyruvate and fermentation rate is shown in Fig. 1, which represents the results of an experiment in which 10 m-moles glucose were completely fermented. Inorganic phosphate (intracellular, the medium gave only trace values throughout) was determined, using an adaptation of the method of Berenblum & Chain (1938), applied to TCA extracts obtained as described under 'Methods'. The results of two separate experiments (Fig. 1) show that the onset of phosphorylation processes was considerably more rapid than the overall fermentation, and suggest that rate control in the first 30 min. of this fermentation was due to enzyme systems intermediate between fructose 1:6-diphosphate and pyruvate. Fig. 2 shows that exhaustion of external glucose (determined, after centrifuging off the yeast, by the method of Heidt, Southam, Benedict & Smith, 1949) brought about a rapid fall in intracellular pyruvate, the level of which, however, was still appreciable long after the disappearance of glucose. The excretion of pyruvate into the medium also stopped when all the glucose had disappeared, and the level of pyruvate outside the cell began slowly to decline.

The quantitative relation between fermentation rate and intracellular pyruvate was examined by making six sets of measurements of rate and pyruvate level, at 15 or 30 min. intervals, on each of six anaerobic fermentations. The same master sample of yeast was used in the first five experiments: the conditions of fermentation were not, however, the same. Thus thirty-six determinations of rate and intracellular pyruvate were obtained, the rate varying from 10 to 53 μmoles/min./g. and the

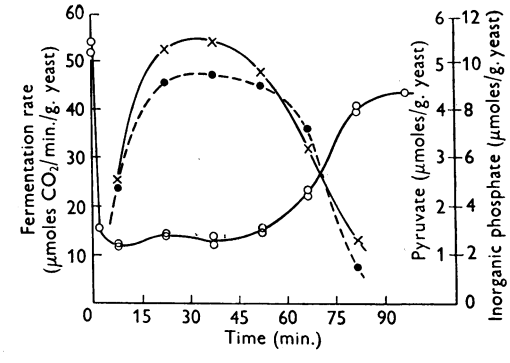


Fig. 1. Intracellular pyruvate (×), fermentation rate (●), and inorganic phosphate (○). 5 g. yeast, 10 m-moles glucose/100 ml., succinate buffer pH 4·1.

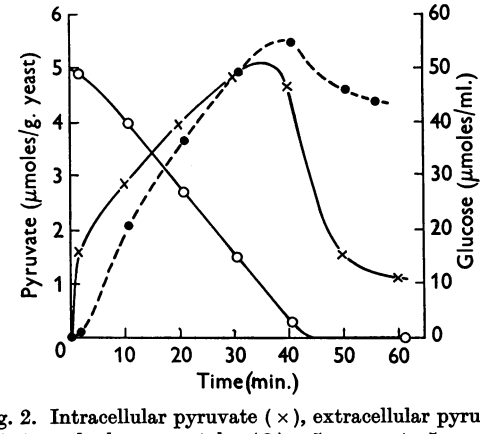


Fig. 2. Intracellular pyruvate (×), extracellular pyruvate (●), and glucose uptake (○). 5 g. yeast, 5 m-moles glucose/100 ml.

pyruvate from 1 to 13 μmoles/g. Some of these measurements were, however, discarded: those made 82·5 min. after initiation of glucose fermentation were unsatisfactory because of the high extracellular pyruvate (for then determination of the intracellular level by difference became inaccurate), while at 7·5 min. the pyruvate measurements were highly accurate but the rate measurements were uncertain, due to rapid variation of rate with time in the initial stage of fermentation.

In four experiments (*a*)–(*d*), glucose was the substrate. The composition of the medium, and the maximum rate (as μmoles $CO_2$/min./g.) recorded, were as follows: (*a*), no addition, 45; (*b*) constant-rate medium described under 'Methods', 53; (*c*) as (*b*), but KCl replaced $KH_2PO_4$ (phosphate-free medium), 47; (*d*) succinate buffer pH 4·1, 0·001 M sodium arsenate, 16. Arsenate is particularly suitable as an inhibitor, since the fermentation rate was found to be insensitive to changes in arsenate concentration between $10^{-4}$ and $5 \times 10^{-3}$ M, and the effect of the inhibitor was therefore highly reproducible. Low values of rate and pyruvate were recorded also in experiment (*e*), in which the yeast fermented maltose in succinate buffer. Pyruvate varied from 1·2 to 2·3 μmoles/g. whilst the rate changed from 10 to 21 μmoles $CO_2$/min./g. In maltose fermentation the rate increased slowly with time, owing to formation of the adaptive enzyme maltase.

Examination of the results showed that the intracellular pyruvate increased more rapidly than fermentation rate. This is reminiscent of the effect produced when an enzyme becomes saturated by its substrate (see, for example, Van Slyke, 1942). Fig. 3 shows the application of the simple Michaelis-Menten equation to the results of Expts. (*a*)–(*d*) (glucose fermentation). The form

$$S/v = S/V_{max.} + K_m/V_{max.}$$

is used, as this emphasizes high substrate values (Hofstee, 1952) which form the bulk of the data. The maximum fermentation rate, $V_{max.}$, derived from the slope of the straight line fitted to the plot of $S/v$ against $S$ by the method of least squares, is 65 μmoles $CO_2$/min./g. yeast; half-maximum rate is produced at an intracellular pyruvate level of $S = K_m = 3·1$ μmoles/g.

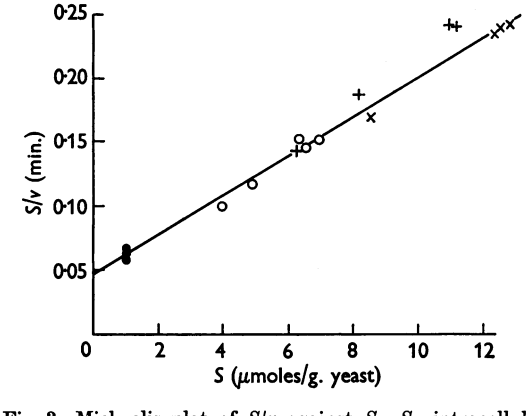


Fig. 3. Michaelis plot of $S/v$ against $S$. $S$ = intracellular pyruvate (μmoles/g. yeast); $v$ = fermentation rate (μmoles $CO_2$/min./g. yeast). ○, data of Expt. (*a*) (see text); ×, Expt. (*b*); +, Expt. (*c*); ●, Expt. (*d*).

Experiment (*f*), which used a different sample of D.C.L. baker's yeast, showed that the time-dependent rate of maltose fermentation enables the values of $V_{max.}$ and $K_m$ to be derived from measurements made between 60 and 240 min. in a single fermentation, though less reliably, since the maximum rate obtained is less than that with glucose in the same medium. Here $V_{max.} = 63$ μmoles $CO_2$/min./g.; $K_m = 2·8$ μmoles pyruvate/g.

*Pyruvate levels in various fermentation systems*

Once the relation between the intracellular level of pyruvate and the fermentation rate has been established, the fermentation rate, or what amounts to the same thing, the velocity of the yeast carboxylase system, may be derived from pyruvate measurements. During the present investigation, pyruvate levels during the first 30 min. of fermentation were measured in a number of systems; usually 20 ml. of the fermenting suspension contained in a 1 in. diameter Pyrex tube were stirred (at 30°) by bubbling gas ($N_2$, $O_2$, $CO_2$). The rate of $CO_2$ production was not measured, though an approximate idea of it could be derived from the rate of glucose uptake. Rate measurements made by the $CO_2$ production method described are averages over at least 10 min., and are valueless when the rate is rapidly changing with time, as it does during the first 30 min. of anaerobic fermentation, with the yeast we have used. Pyruvate measurements can be made, however, as early as 1 min. after mixing the yeast and glucose. An expected error in the extracellular value, due to the time taken in centrifuging, did not arise—since the extracellular pyruvate was in fact negligible. At this time, in one experiment, the intracellular pyruvate had increased from 0·7 μmole/g. before addition of glucose to 1·5. At 5 min. the value was 4, after which the level rose by about 1 μmole/g. every 5 min. Since the rate/pyruvate relation is hyperbolic, these figures suggest an initially rapid rise in rate, the acceleration decreasing with time.

An experiment in which glucose was metabolized aerobically is interesting. If the hyperbolic relation between pyruvate level and the velocity of the carboxylase system, derived from studies of

anaerobic fermentation, is assumed to apply here, then the pyruvate level of 1·6 μmoles/g. observed corresponds to a carboxylase rate of about 20 μmoles $CO_2$/min./g., or 0·3 of the rate in anaerobic fermentation. The glucose uptake was about 0·35 of that in the anaerobic system. This strongly suggests that, under the experimental conditions employed, glucose was metabolized by the anaerobic system as far as acetaldehyde (cf. Weinhouse, Millington & Lewis, 1948). Further, the suggestion of Myrbäck & Vallin (1944) that the Pasteur effect in yeast is due to inactivation (as the disulphide) of cocarboxylase receives no support from this experiment, for no accumulation of pyruvate was demonstrable. Replacing nitrogen by $CO_2$ lowered the intracellular pyruvate during the first 30 min. of fermentation. Adding bicarbonate to give a higher pH increased the extracellular pyruvate production, though the intracellular levels were not affected proportionately.

The strain of *Saccharomyces cerevisiae* used in the manufacture of D.C.L. baker's yeast was, in some preliminary experiments, grown in the laboratory under sterile conditions. Eighteen-hour cultures were employed, grown at 30° in the synthetic medium of Olson & Johnson (1949). The intracellular pyruvate level (1–2 μmoles/g.) was considerably lower, during fermentation, than in comparable experiments using pressed yeast; and this was irrespective of whether the medium contained thiamine or not. This suggests (1) that the yeast strain is capable of cocarboxylase synthesis without an external supply of thiamine, and (2) that the high intracellular pyruvate level of pressed yeast (or at least of the particular type we have used) is not a feature of the strain of organism, but due to the method of manufacture.

## DISCUSSION

We have shown that the velocity of the carboxylase system in the living yeast cell (which in anaerobic fermentation is identical with the rate of carbon dioxide production) depends on the intracellular pyruvate level in the same way as, *in vitro*, the velocity of a simple enzyme reaction depends on substrate concentration. This relation may be treated as a purely empirical one. Even so, the results demonstrate that in a favourable case the methods of enzyme kinetics may be applicable to the living cell. Again, the calculation of carboxylase activity from pyruvate measurement, as applied for example to the preliminary experiment on aerobic glucose metabolism reported in the previous section, does not necessarily depend on a particular interpretation of the carboxylase–pyruvate relation. The apparent 'saturation' of the yeast carboxylase with pyruvate at higher fermentation rates may be interpreted in several ways. For example, the carboxylase may be inhibited by some substance (e.g. acetaldehyde) which increases in concentration as fermentation rate increases. The simplest hypothesis is, however, that referred to in the Introduction; namely, that the intracellular pyruvate, as defined in the section on Methods, is a direct measure of pyruvate concentration at the site of carboxylase action. It is important to note that the accuracy of the data obtained does not permit any deduction based on the exact form of the mathematical relation between fermentation rate and pyruvate level.

The spatial distribution of the carboxylase system in the yeast cell is not known. Chantrenne (1944) found carboxylase activity in yeast extracts was not associated with particulate material. However, according to Michaelis (1949), the kinetics of an enzyme system are similar whether the enzyme is in homogeneous solution or in particulate form; consequently, it is not the distribution of carboxylase within the yeast cell that is important, but the distribution of the substrate pyruvate. If this is assumed to be equally distributed throughout the cell, i.e. diffusion rates are taken to be rapid in comparison with the rate of enzyme action, then the intracellular pyruvate level, as μmoles/g. yeast, may be transformed into substrate concentration if the intracellular water content of the yeast is known. According to Lindenberg, Monique & Guillemet (1949) baker's yeast dispersed in water contains 66% intracellular water, which falls to 63·5% when the yeast is suspended in 0·1 M-NaCl. Thus, on the assumption that we have actually measured substrate concentration and carboxylase velocity, the results may be briefly stated as follows: for the yeast used in the series of experiments (*a*)–(*e*) described above the *in vivo* activity (maximum velocity) of carboxylase was 65 μmoles pyruvate (or $CO_2$)/min./g. yeast, and the Michaelis constant 3 μmoles/g. or about $4 \times 10^{-3}$ M pyruvate. [Green, Herbert & Subrahmanyan (1941) found the Michaelis constant of purified yeast carboxylase in citrate buffer, pH 6, to be $3 \times 10^{-2}$ M. An earlier report (Hägglund & Rosenquist, 1927) stated that pyruvate concentration had no effect on the velocity of $CO_2$ production by a crude yeast preparation when varied between 0·03 and 0·50 M, but at 0·01 M activity was markedly decreased. Wheat germ carboxylase, which has been obtained in a highly purified state by Singer & Pensky (1952), is half-saturated at $3·6 \times 10^{-3}$ M pyruvate (succinate buffer pH 6, 30°).]

The question of whether the rate of alcoholic fermentation can be said to be 'limited' by one or other enzyme system has been frequently discussed. With such a complex process as fermentation by living yeast, in which so many factors are beyond

experimental control, no general satisfactory definition of 'limiting enzyme' can be made. In the case of an enzyme catalysing the irreversible decomposition of a single substrate, it will clearly limit the overall rate of fermentation when it is saturated with respect to its substrate. When the yeast we have studied ferments excess glucose at 30° carboxylase very nearly meets this requirement: according to the simple interpretation of the results we have suggested, this enzyme system is about 80% saturated by 12 $\mu$moles pyruvate/g. yeast. It should be noted that this does not imply that raising the activity of the carboxylase system would necessarily result in an increased fermentation rate: what is implied, however, is that no substantial rate increase is possible unless the activity of intracellular carboxylase is increased through synthesis of specific protein, or of coenzyme, or even possibly by alteration of physico-chemical properties of the enzyme milieu, such as pH.

## SUMMARY

1. The intracellular pyruvate level of yeast has been measured as the difference between the total pyruvate extracted from a suspension by trichloroacetic acid and that excreted into the medium.

2. The relation between fermentation rate and intracellular pyruvate resembles that between the velocity of an enzyme reaction and substrate concentration.

3. The velocity of the carboxylase system in metabolizing yeast suspensions may therefore be deduced from pyruvate measurement when direct measurement by determination of carbon dioxide output is not feasible.

4. A possible interpretation of the results as a direct determination of the kinetics of the carboxylase system *in vivo* is given.

5. The limitation of fermentation rate, in the yeast studied, by carboxylase is discussed.

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# Studies on Yeast Metabolism

## 4. THE EFFECT OF THIAMINE ON YEAST FERMENTATION

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Since the discovery of the acceleration of alcoholic fermentation by thiamine (Schultz, Atkin & Frey, 1937*a*) and the development by these authors (see their review in Anderson, 1946) of their fermentation test, several attempts have been made to establish the nature of the effect. The problem is complicated by the fact that not all yeasts respond to thiamine in the same way, varying periods of incubation being required (e.g. Laser, 1941).

According to Sperber & Renval (1941) thiamine is taken up by yeast in two phases; a rapid absorption is followed by a slower conversion of absorbed thiamine into cocarboxylase. Westenbrink, Steyn-Parvé & Veldman (1947) also studied the conversion of thiamine into cocarboxylase by living yeast, but found that although 200–300 $\mu$g. of cocarboxylase could accumulate per gram of yeast, no increase in the enzyme carboxylase could be demonstrated.